ELSEVIER

Virus Research 58 (1998) 45–52

Virus Research

Matrix protein gene nucleotide and predicted amino acid sequence demonstrate that the first US avian pneumovirus isolate is distinct from European strains

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Received 30 June 1998; received in revised form 7 August 1998; accepted 7 August 1998

Abstract

Avian pneumovirus (APV) is the etiological agent of turkey rhinotracheitis (TRT). Outbreaks of TRT first occurred in the US during May, 1996 and continued through June, 1997. This is the first report of these virus types in the US that was previously considered exotic to the US and Canada. The US isolate, APV/CO, was replicated in chick embryo fibroblasts (CEF) and poly-A RNA from APV/CO infected CEF cells was purified for cDNA synthesis. Degenerate oligonucleotide primers were used to amplify nucleotide sequences coding for the matrix (M) protein gene. Although the type A and B European APV M genes share 75% identity in their coding sequences, they have only 60% identity with the US APV/CO M protein gene. Predicted M proteins of European APV type A and B isolates share 89% identity in their amino acid sequence. However, the predicted M protein of APV/CO has only 78% identity with APV type A and 77% identity with APV type B protein sequences. Phylogenetically the US APV/CO isolate separates as a unique virus relative to European APV type A and B strains that cluster together. Sequence information for the APV/CO M protein gene and predicted amino acids of the M protein confirm the unique nature of this isolate compared to its European counterparts. This correlates with the inability to serologically detect the US APV/CO isolate using diagnostics based on European viruses.

Keywords: Turkey rhinotracheitis virus; Matrix protein; Mononegavirales; Viral evolution; Avian pathogen; Emerging diseases; Veterinary virology

1. Introduction

Avian pneumovirus (APV) causes turkey rhinotracheitis (TRT) and swollen head syndrome (SHS) of chickens that is usually accompanied by

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0168-1702/98/$ - see front matter
PII S0168-1702(98)00098-7

secondary bacterial infections which increase mortality. Clinically, in turkeys the disease is similar to *Bordetella avium* infections and is primarily respiratory with loss of egg production. In chickens, APV infections may be subclinical with SHS not developing. The virus was first reported in South Africa during the early 1970s and since then has been isolated in Europe, Israel and Asia. Clinical signs of birds infected with APV are primarily respiratory including rales, sneezing and nasal discharge (Jones, 1996; Alexander, 1997). In experimentally infected chickens and turkey poults, viral antigen is detected primarily in the turbinate cilia, trachea and lung epithelial cells (Majo et al., 1995, 1996, 1997). Extensive replication of APV in turbinates causes severe rhinitis that facilitates infection by secondary bacterial agents (Jones et al., 1988; Majo et al., 1996).

Pneumoviruses are members of the family *Paramyxoviridae* that contain a nonsegmented negative-strand RNA genome of approximately 15 kb in length. Viruses related to APV include human, bovine, ovine and caprine respiratory syncytial viruses and pneumonia virus of mice. Pneumoviruses generally encode ten genes, versus six or seven as in the other paramyxoviruses. Although pneumoviruses have an F protein that promotes cell fusion, these virus types do not hemagglutonate, nor do they have neuraminidase activity in their G attachment protein. This is an important distinguishing characteristic from the other paramyxoviruses (Collins et al., 1996).

Initial classification of European APV isolates was based on molecular characterization of the virion (Collins et al., 1986; Collins and Gough, 1988), electrophoretic mobility of viral proteins (Ling and Pringle, 1988) and a number of mRNA species detected in viral infected cells (Cavanagh and Barrett, 1988). Sequence information for the N (Li et al., 1996), P (Ling et al., 1995), M (Yu et al., 1992b; Randhawa et al., 1996a), F (Naylor et al., 1998; Yu et al., 1991), M2 (Yu et al., 1992a), SH (Ling et al., 1992), G (Ling et al., 1992; Juhasz and Easton, 1994) and L (Randhawa et al., 1996b) genes have been published for several European APV isolates. The sequence of each gene in every case is most similar to other members of the *Pneumovirus* genus. The putative gene order of APV (3′-N-P-M-F-M2-SH-G-L-5′) is unlike its mammalian counterparts (3′-NS1-NS2-N-P-M-SH-G-F-M2-L-5′), wherein the SH and G genes are located 5′ to the M2 gene (Ling et al., 1992). The extreme 3′ and 5′ ends of an European APV isolate's genome were determined which established that the NS1 and NS2 genes are absent in these viruses (Randhawa et al., 1997). This is also distinct from the mammalian pneumoviruses and along with a smaller L gene results in APV having a genome of only 13.3 kb (Randhawa et al., 1996b). Consequently, since APV lacks NS1 and NS2 genes, but has a M2 gene with structural characteristics like other pneumoviruses, it has been designated a new representative of the *Pneumovirinae* (Pringle, 1996).

In February, 1997 the National Veterinary Services Laboratory, Animal Plant Health Inspection Services (NVSL, APHIS) of USDA reported the first official APV isolation from commercial turkeys in Colorado (APV/CO). Outbreaks of TRT-like respiratory disease first occurred during May, 1996 and continued through June, 1997. During the first 10 months of the US outbreak, it was not possible to detect virus serologically due to lack of cross-reactivity to the US APV isolate with reagents produced in Europe. Consequently, an ELISA was developed by NVSL using purified, inactivated APV/CO as an antigen. Serological evidence of APV infection was subsequently also demonstrated in North-Central US turkey flocks following reports of severe respiratory disease of unknown etiology in Minnesota during March, 1997. Mortality due to APV ranged from zero to as high as 30% when accompanied by bacterial infections with condemnations due to airsacculitis (Kleven, 1997). Absence of serological reactivity by APV/CO-infected birds using previous APV isolates clearly demonstrates emergence of new North American strains of this virus group. To determine if the US isolate was a new APV type, the M protein gene was cloned, sequenced and phylogenetic analysis conducted.

The APV/CO isolate (D. Senne, NVSL, APHIS, USDA, personal communication) was propagated in chicken embryo fibroblast (CEF) cells using standard techniques (Wyeth and Alex-

ander, 1989; Alexander, 1997). Following replication in CEF cells, RNA was purified by guanidinium extraction (Chirgwin et al., 1979) of infected cells and ultracentrifugation through CsCl (Glisin et al., 1974). The 5′ region of conserved nucleotide sequence among APV M protein genes (Yu et al., 1992b; Randhawa et al., 1996a) were analyzed by the PRIMER2 (Scientific and Educational Software, Stateline, PA) computer program to obtain an oligonucleotide primer. Polyadenylated RNA was purified (Aviv and Leder, 1972) and cDNA synthesized by reverse transcription–polymerase chain reaction (Belyavsky et al., 1989) utilizing an oligo-dT primer ($GGGAGGCCCCT_{15}$) with a conserved APV 5′ M gene primer (GGGGACAAGTIAAIATGGAGTC). Amplified products were cloned using TA cloning systems (Mead et al., 1991; Seal, 1996) according to methods of the manufacturer (Invitrogen, San Diego, CA; Promega, Madison, WI). The ligated cDNA was introduced into *E. coli* using standard transformation buffer and plated on media with ampicillin (Hanahan, 1985).

Double-stranded sequencing (Sanger et al., 1977) with *Taq* polymerase (Applied Biosystems) and fluorescently labelled dideoxynucleotides was performed with an automated sequencer (Smith et al., 1986). Six independent APV/CO M protein gene clones from two separate amplification and cloning reactions were sequenced. Nucleotide sequence editing, prediction of amino acid sequences, and protein computer structure predictions were completed using the DNASTAR (Madison, WI) and GeneWorks 2.3 programs (Intelligenetics, Mountain View, CA). Alignments were performed using the CLUSTALW method (Thompson et al., 1994). Nucleotide sequence analysis, including determination of synonymous and nonsynonymous substitutions (Nei and Gojobori, 1986), was completed using the molecular evolutionary genetics analysis system (MEGA; Kumar et al., 1993). To determine relationships among APV isolates and how protein sequence information relates to current designations, analysis was performed by phylogenetic analysis using parsimony (PAUP; Swofford, 1989) with the APMV1/turkey(ND)/US/92 M protein (Seal, 1996) as an outgroup (Smith, 1994).

An 877-bp insert was obtained following amplification and cloning of the putative APV/CO M gene. The cloned DNA sequence contained one major ORF from positions 14 to 775 and the 5′ terminus included the APV mRNA start site sequence GGGACAAGT. This gene codes for a protein of 254 amino acids with a predicted molecular weight of 27651 and an isoelectric point of 8.19 with a charge of 2.16 at pH 7. This is in agreement with what was reported for the M protein gene of APV subtypes A and B (Yu et al., 1992a; Randhawa et al., 1996a). The APV/CO M protein's relative mobility during polyacrylamide gel electrophoresis of purified virus confirmed the predicted molecular weight (data not shown). The TAA stop codon of APV/CO was shared with APV isolate 2119/Italy/1988, a representative of subtype B (Randhawa et al., 1996a,b), while APV type A isolate 3BV/UK/1985 had a TGA stop codon (Yu et al., 1992b) in its M protein gene. The number of nucleotides in the M protein gene mRNA 3′ noncoding region was not equal for all three isolates. This further substantiates that APV, like other pneumoviruses, does not require the genome to contain a number of nucleotides divisible by six (Randhawa et al., 1997).

The APV/CO isolate M protein gene coding sequence shared 60% identity to the APV type A and B genes. In contrast, the M protein gene coding sequences of APV type A and B are 75% similar, indicating that the US isolate is substantially different from European strains. Among all three M protein genes the viruses share a 62% nucleotide sequence identity. The ratios of nonsynonymous to synonymous changes is less than one when comparing the M protein gene coding sequences among all three APV isolates. However, the ratio for comparison of the two European isolates is 0.24, while APV/CO has a ratio of 0.5 and 0.57 as compared to APV subtypes A and B, respectively. The ratios are less than one, which may indicate purifying selection has occurred (Kimura, 1977). However, the greater number of nonsynonymous changes in the APV/CO M protein gene relative to European isolates may indicate some positive selection. Also, variable portions of the M protein gene have nonsynonymous to synonymous ratios greater than one.

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APV/CO      ....LV.... .........T ......QL.. R......... TN..PT...E
APV/A       .......... .......... ..I...S... T......... ..........
APV/B       .......... .......... ......N... K......... ..........
Consensus   MESYIIDTYQ GVPYTAAVQV DLVEKD.NPA .LTVWFPLFQ SSTPAPVLLD  50

APV/CO      .....T...L ....QN..I. K...S..... ......S.D. ..S....D..
APV/A       .......... .......... ....A..... ........A. ..........
APV/B       .......... ..V....... ....T..... ........S. ...A......
Consensus   QLKTLSITTQ YTASPEGPVL QVNA.AQGAA MSALPKKF.V SAAVALDEYS 100

APV/CO      ....DK.... ELK......M ........F. NSAKA..K.. .........L
APV/A       R....T.... ...SI..... .........M .DVRS..... ..........
APV/B       ..D..V.... .......... .......... .NMNT..... ..........
Consensus   KLEFG.LTVC DVRAVYLTTL KPYGMVSKIV T....VGRKT HDLIALCDFI 150

APV/CO      .L........ .....S.... E......... .......... ..........
APV/A       .I.....I.. .......... .......... .......... ..........
APV/B       .M.R.I.... .......... .......... .......... ..........
Consensus   D.EKGVPVTI PAYIKAVSIK DSESATVEAA ISGEADQAIT QARIAPYAGL 200

APV/CO      .M........ .........V .......A.. ....ISR..R N.S..G....
APV/A       .......... .........M .......... .......... ..........
APV/B       ..L.A..... ...R.....T .......... .......... .........I
Consensus   ILIMTMNNPK GIFKKLGAG. QVIVELGPYV QAESLGKICK TWNHQRTRYV 250

APV/CO      ....
APV/A       .R..
APV/B       ....
Consensus   LKSR                                                  254
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Fig. 1. Comparative amino acid sequence alignment of matrix proteins among avian pneumovirus isolates. The US avian pneumovirus (APV) isolate is represented by APV/CO, while previously identified European subtypes are designated APV/A and APV/B as cited in the text. Amino acid sequence differences are denoted by the single-letter code with the consensus below.

For example, nucleotide sequences from positions 387 to 405 encoding predicted amino acid residues 129–135 (Fig. 1) had a greater number of nonsynonymous changes. The ratios were 1.56 and 1.84 when comparing APV/CO to APV subtypes A and B, respectively. The ratio of nonsynonymous to synonymous changes was 1.85 between the two European isolates. This indicates that, similar to other viral proteins (Ina and Gojobori, 1994; Seibert et al., 1995), certain variable regions may be undergoing natural selection.

Alignment of the predicted M protein amino acid sequences demonstrates that heterogeneity occurs throughout the APV/CO protein relative to APV subtypes A and B (Fig. 1). Predicted APV subtype A and B proteins share 89% identity. However, the APV/CO M protein shares only 78 and 77% identity with the subtype A and B proteins, respectively. The majority of amino acid substitutions were, however, conserved. This is supported by the fact that M proteins of all three APV isolates contain more hydrophobic amino acids (43%) relative to polar residues (32%). Charged amino acids make up 25% of the M protein of APV and contribute to its somewhat basic nature.

A variable region from amino acid residues 129 to 135 is present among all APV M proteins (Fig. 1). This area abuts and partially includes a portion of the protein that is hydrophilic (Kyte and

Doolittle, 1982) with a high surface probability (Emini et al., 1985) relative to the remainder of the M protein. It also corresponds to a region of the gene that contains a greater number of non-synonymous changes relative to synonymous nucleotide substitutions. A 14-residue hydrophobic region of the M protein, surrounding amino acid 200 contained within respiratory syncytial virus and APV (Yu et al., 1992b), is relatively conserved among all three isolates (Fig. 1).

Phylogenetic analysis of M protein amino acid sequences using parsimony demonstrates that avian pneumoviruses form a single clade relative to their mammalian counterparts. However, within the avian branch, APV/CO separates from the APV subtypes A and B cluster (Fig. 2). The same relationship among APV isolates was obtained using the neighbor-joining method (Saitou and Nei, 1987) and/or nucleotide coding sequences of the M protein gene (data not shown). This relationship among avian pneumoviruses is supported by high bootstrap confidence levels following 2000 replications (Hedges, 1992). Among paramyxoviruses the matrix protein is the second most highly conserved protein (Rima, 1989), following the polymerase which is frequently used for phylogenetic analysis (Tordo et al., 1992). Consequently, these data for deduced M proteins of APV isolates indicate the US APV/CO isolate is distinct from European viruses.

Based on G protein gene nucleotide and predicted amino acid sequences, two APV subgroups, designated A and B, were identified (Juhasz and Easton, 1994). Group A viruses included isolates from the UK, while group B viruses included isolates from Spain, Italy and Hungary. The original French APV isolate previously considered a type A virus (Collins et al., 1993; Juhasz and Easton, 1994) was proven to also belong to the type B group (Naylor et al., 1997). The G proteins were 98.5–99.7% similar among groups, but shared only 38% identity between the two APV clusters. This correlates with earlier data demonstrating that various APV isolates were antigenically similar, but could be separated serologically into two separate groups (Collins et al., 1993; Cook et al., 1993). This relationship was further confirmed by sequence analysis of the F protein gene (Naylor et al., 1998) and the more conserved M protein gene (Randhawa et al., 1996a). This could account for discrepancies using different antigens for serology (Eterradossi et al., 1992). Unfortunately, no sequence information is cur-

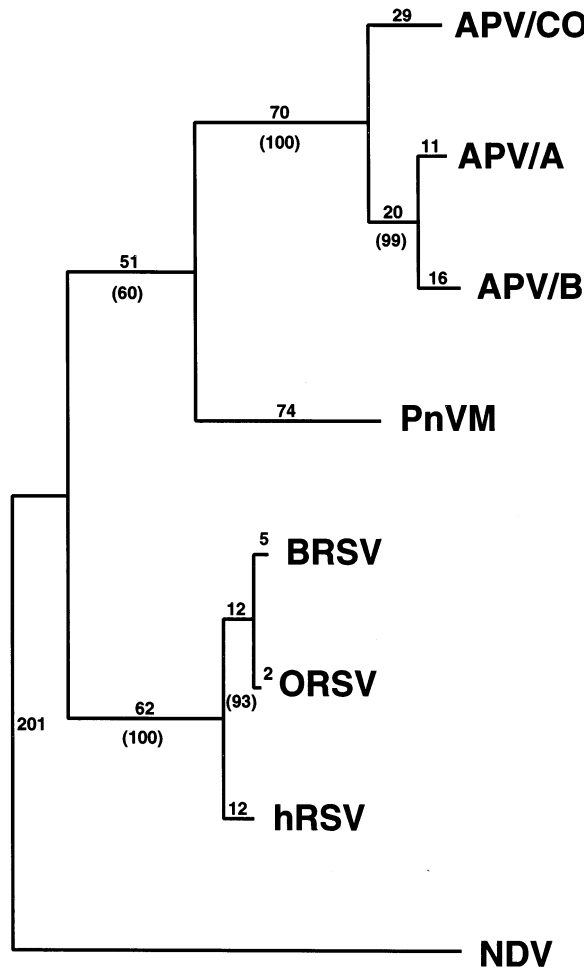

Fig. 2. Phylogenetic relationships among pneumoviruses based on matrix protein amino acid sequences. Following alignment an unrooted phylogram was generated by maximum parsimony analysis. Absolute distances are listed above each branch with bootstrap confidence levels in parentheses below. Abbreviations are PnVM for pneumonia virus of mice (Easton and Chambers, 1997); hRSV is human respiratory syncytial virus (Satake and Venkatesan, 1984), ORSV and BRSV are ovine (Alansari and Potgeiter, 1994) and bovine (Samal and Zamora, 1991) respiratory syncytial virus; APV/A (Yu et al., 1992b) and APV/B (Randhawa et al., 1996a) are European subtypes A and B, while APV/CO represents the US avian pneumovirus isolate reported in the text.

rently available for recent Japanese APV isolates (Tanaka et al., 1995, 1996). The APV/CO US isolate is serologically different from European isolates (Kleven, 1997) and this relationship was further confirmed by sequence analysis of the M protein gene reported herein.

Acknowledgements

Gratitude is extended to the excellent technical assistance of Joyce Bennett for nucleotide sequencing. The author also thanks the Molecular Genetics Instrumentation Facility at the University of Georgia for oligonucleotide synthesis. Appreciation is extended to Corrie Brown, John Crawford, Daniel J. King, Steve Kleiboeker, Mike Perdue, Steve Poet, Julia Ridpath, Stacey Schultz-Cherry and David Suarez for helpful critiques of the manuscript. Nucleotide sequence for the M protein gene of APV/CO is submitted to GenBank with accession no. AF072682. These investigations were supported by USDA, ARS, CRIS project number 6612-32000-015-00D-085.

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